

# Targeting Platelet–Leukocyte Interactions: Identification of the Integrin Mac-1 Binding Site for the Platelet Counter Receptor Glycoprotein Ib $\alpha$

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## Abstract

The firm adhesion and transplatelet migration of leukocytes on vascular thrombus are dependent on the interaction of the leukocyte integrin Mac-1 ( $\alpha_M\beta_2$ , CD11b/CD18) and the platelet counter receptor glycoprotein (GP) Ib $\alpha$ . Previous studies have established a central role for the I domain, a stretch of  $\sim$ 200 amino acids within the  $\alpha_M$  subunit, in the binding of GP Ib $\alpha$ . This study was undertaken to establish the molecular basis of GP Ib $\alpha$  recognition by  $\alpha_M\beta_2$ . The P<sup>201</sup>–K<sup>217</sup> sequence, which spans an exposed loop and amphipathic  $\alpha_4$  helix in the three-dimensional structure of the  $\alpha_M$ I domain, was identified as the binding site for GP Ib $\alpha$ . Mutant cell lines in which the  $\alpha_M$ I domain segments P<sup>201</sup>–G<sup>207</sup> and R<sup>208</sup>–K<sup>217</sup> were switched to the homologous, but non-GP Ib $\alpha$  binding,  $\alpha_L$  domain segments failed to support adhesion to GP Ib $\alpha$ . Mutation of amino acid residues within P<sup>201</sup>–K<sup>217</sup>, H<sup>210</sup>–A<sup>212</sup>, T<sup>213</sup>–I<sup>215</sup>, and R<sup>216</sup>–K<sup>217</sup> resulted in the loss of the binding function of the recombinant  $\alpha_M$ I domains to GP Ib $\alpha$ . Synthetic peptides duplicating the P<sup>201</sup>–K<sup>217</sup>, but not scrambled versions, directly bound GP Ib $\alpha$  and inhibited  $\alpha_M\beta_2$ -dependent adhesion to GP Ib $\alpha$  and adherent platelets. Finally, grafting critical amino acids within the P<sup>201</sup>–K<sup>217</sup> sequence onto  $\alpha_L$ , converted  $\alpha_L\beta_2$  into a GP Ib $\alpha$  binding integrin. Thus, the P<sup>201</sup>–K<sup>217</sup> sequence within the  $\alpha_M$ I domain is necessary and sufficient for GP Ib $\alpha$  binding. These observations provide a molecular target for disrupting leukocyte–platelet complexes that promote vascular inflammation in thrombosis, atherosclerosis, and angioplasty-related restenosis.

Key words: inflammation • leukocytes • platelets • adhesion • receptors

## Introduction

Adhesive interactions between vascular cells play important roles in orchestrating the inflammatory response. Recruitment of circulating leukocytes to vascular endothelium requires multistep adhesive and signaling events, including selectin-mediated attachment and rolling, leukocyte activation, and integrin-mediated firm adhesion and diapedesis that result in the infiltration of inflammatory cells into the blood vessel wall (1). Firm attachment is mediated by members of the  $\beta_2$  integrin family, LFA-1 ( $\alpha_L\beta_2$ , CD11a/CD18), Mac-1

( $\alpha_M\beta_2$ , CD11b/CD18), and p150,95 ( $\alpha_M\beta_2$ , CD11c/CD18), which bind to endothelial counter ligands (e.g., intercellular adhesion molecule [ICAM]-1; 2), endothelial-associated extracellular matrix proteins (e.g., fibrinogen; 3), or glycosaminoglycans (4).

Leukocyte recruitment and infiltration also occur at sites of vascular injury where the lining endothelial cells have been denuded and platelets and fibrin have been deposited.

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*Abbreviations used in this paper:* BCECF AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; GP, glycoprotein; GST, glutathione S-transferase; ICAM, intercellular adhesion molecule; MFI, mean fluorescence intensity; NIF, neutrophil inhibitory factor; RU, resonance unit; sGP Ib $\alpha$ , soluble extracellular region of GP Ib $\alpha$  (i.e., glycolalicin); vWf, von Willebrand factor.

A similar sequential adhesion model of leukocyte attachment to and transmigration across surface-adherent platelets has been proposed (5). The initial tethering and rolling of leukocytes on platelet P-selectin (6) are followed by their firm adhesion and transplatelet migration, processes that are dependent on  $\alpha_M\beta_2$  (5).

Our laboratory has focused on identifying the platelet counter receptor for  $\alpha_M\beta_2$ . Evaluation of the structural features of integrins provides insight into candidate platelet counter receptors for  $\alpha_M\beta_2$ . Integrins are heterodimeric proteins composed of one  $\alpha$  and one  $\beta$  subunit. A subset of integrin  $\alpha$  subunits, including  $\alpha_M$ , contains an inserted domain (I domain) of  $\sim 200$  amino acids that is implicated in ligand binding (7–9) and strikingly similar to the A domains of von Willebrand factor (vWf; 10), one of which, A1, mediates the interaction of vWf with its platelet receptor, the glycoprotein (GP) Ib-IX-V complex. Because of the similarity of the vWf A1 domain and the  $\alpha_M$ I domain, we hypothesized that GP Ib $\alpha$  might also be able to bind  $\alpha_M\beta_2$  and reported that GP Ib $\alpha$  is indeed a constitutively expressed counter receptor for  $\alpha_M\beta_2$  (11). Furthermore, under the conditions used in these studies, the predominant interaction between neutrophils and platelets appeared to be between  $\alpha_M\beta_2$  and GP Ib $\alpha$  (11).

The  $\alpha_M$ I domain contributes broadly to the recognition of ligands by  $\alpha_M\beta_2$  (12) and specifically to the binding of GP Ib $\alpha$  (11). This region has been implicated in the binding of ICAM-1 (13), iC3b (14), fibrinogen (12, 13), and neutrophil inhibitory factor (NIF; 15), as well as GP Ib $\alpha$ . Previous studies suggested that overlapping, but not identical, sites are involved in the recognition of iC3b, fibrinogen, and NIF (16, 17). Although the binding sites for iC3b, NIF, and fibrinogen in the  $\alpha_M$ I domain have been mapped extensively (18–23), the recognition site for GP Ib $\alpha$  is unknown.

In this study, we have localized the binding site for GP Ib $\alpha$  within the  $\alpha_M$ I domain. The strategy developed was based on the differences in the binding of GP Ib $\alpha$  to the  $\alpha_M$ I and  $\alpha_L$ I domains and involved several independent approaches, including screening of mutant cells, synthetic peptides, site-directed mutagenesis, and gain in function analyses. The binding site for GP Ib $\alpha$  was localized within the segment  $\alpha_M$ (P<sup>201</sup>–K<sup>217</sup>). The grafting of two amino acids within this segment into the  $\alpha_L$ I domain converted it to a GP Ib $\alpha$ -binding protein. Thus, a small segment that has a defined structure within the  $\alpha_M$ I domain is necessary and sufficient for GP Ib $\alpha$  binding.

## Materials and Methods

**Materials.** The soluble extracellular region of GP Ib $\alpha$  (sGP Ib $\alpha$ ; i.e., glycolocalin) was purified as we previously reported (11). Human fibrinogen depleted of plasminogen, vWf, and fibronectin was purchased from Enzyme Research Laboratories.

The CD11/CD18 mAbs used included the following: LPM19c, directed to the  $\alpha_M$ I domain (provided by K. Pulford, Radcliffe, Oxford, United Kingdom; 12); OKM1, directed to the  $\alpha_M$  subunit of human Mac-1 (American Type Culture Collection [ATCC]); M1/70, directed to the  $\alpha_M$  subunit mouse  $\alpha_M\beta_2$

(ATCC; 24); CBRM1/5, an activation-specific  $\alpha_M$  reporter antibody (provided by T. Springer, Harvard Medical School, Boston, MA; 25); TS1/22, directed to the  $\alpha_L$  subunit of  $\alpha_L\beta_2$  and capable of blocking ICAM-1 binding (ATCC); MEM-83, an activation-specific  $\alpha_L$  reporter antibody (Caltag); and IB4, a blocking mAb directed to the  $\beta_2$  subunit (ATCC). The stimulating CD18 mAb KIM127 (26) was provided by M. Robinson (Celltech Ltd., Slough, United Kingdom). mAb24, a  $\beta_2$  activation reporter antibody (27), was provided by N. Hogg (Imperial Cancer Research Fund, Lincoln's Inn Fields, London, United Kingdom).

Peptides were obtained from the W.M. Keck Biotechnology Resource Center at Yale University. The peptides were diluted in DMSO and stored at  $-80^\circ\text{C}$ .

**Cell Lines and Culture Conditions.** THP-1 monocytic cells (ATCC) were maintained and differentiated with 1 ng/ml TGF- $\beta$ 1 and 50 nM 1,25-(OH)<sub>2</sub> vitamin D3, as previously described (28). 293 cells expressing human  $\alpha_L\beta_2$ ,  $\alpha_M\beta_2$ , or mutant  $\alpha_M\beta_2$  receptors were established and maintained as previously described (16, 20, 23, 29).

**Segment Switches by Site-directed Mutagenesis.** To systematically define the GP Ib $\alpha$ -binding site in  $\alpha_M$ , a homologue-scanning mutagenesis strategy was implemented (20). Accordingly, guided by the crystal structure (8, 9), the hydrated surface of the  $\alpha_M$ I domain was replaced with sequences of the  $\alpha_L$ I domain in segments of 7–11 amino acids. To apply this approach to the  $\alpha_M$ I domain ( $\sim 200$  amino acids), 16 segments were switched (20, 23). Site-directed mutagenesis of the  $\alpha_M$ I domain was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene). The mutations introduced and the mutagenic primers used have been reported (23). The appropriate DNA sequence of the entire I domain (from I<sup>139</sup> to A<sup>332</sup>) was confirmed for each mutant before transferring back into the  $\alpha_M$  subunit cDNA.

**Transient Transfection.** The expression vector pcDNA3.1 (Invitrogen) was used for cloning  $\alpha_M$ ,  $\alpha_L$ , and  $\beta_2$  from human leukocyte cDNA library. 293 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) with 24  $\mu\text{g}$  DNA/vessel for 4 h, according to the manufacturer's instructions. After transfection, the medium was replaced with full growth medium. The functional assays were prepared 48 h after transfection.

**Flow Cytometry.** FACS<sup>®</sup> analyses were performed to assess the expression of wild-type and mutated forms of  $\alpha_M\beta_2$  and  $\alpha_L\beta_2$  on the surface of transfected 293 cells, as previously described (11). Platelet P-selectin expression was assessed using FITC-conjugated AK-4 or isotype control (BD Biosciences).

**Preparation of Neutrophils and Platelets.** Neutrophils from wild-type (Mac-1<sup>+/+</sup>) and Mac-1-deficient (Mac-1<sup>-/-</sup>; 30) C57Bl/J6 mice were harvested and purified from the peritoneal cavity after the intraperitoneal injection of 1 ml sterile 3% thioglycollate broth, as previously described (11).

Venous blood was obtained from volunteers who had not consumed aspirin or other nonsteroidal antiinflammatory drugs for at least 10 d and was anticoagulated with 13 mM trisodium citrate, which also contained 100 nM prostaglandin E<sub>1</sub>. Platelet-rich plasma was prepared by centrifugation at 150 g for 10 min. Gel-filtered platelets were obtained by passage of platelet-rich plasma over a Sepharose-2B column in calcium-free Tyrode's-Hepes buffer containing 100 nM prostaglandin E<sub>1</sub>, as previously described (11).

**Adhesion Assays.** Adherent cells were assayed by loading 293 cells and thioglycollate-elicited murine neutrophils with 1  $\mu\text{M}$  2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester (BCECF AM), according to the manufacturer's instructions (Molecular Probes). 10<sup>5</sup> cells/well were placed in 96-

well microtiter plates coated with 10  $\mu\text{g/ml}$  sGP Ib $\alpha$  or 10  $\mu\text{g/ml}$  fibrinogen and blocked with 0.2% gelatin. Adhesion was stimulated with 20 ng/ml PMA or 5  $\mu\text{g/ml}$  of the  $\beta_2$ -stimulating mAb KIM 127. Plates were washed and adhesion was quantified by measuring the fluorescence of BCECF AM-loaded cells using a Cytofluor II fluorescence microplate reader (PerSeptive Biosystems). The effect of anti- $\alpha_M$  mAb on adhesion was assessed by preincubating cells with 10  $\mu\text{g/ml}$  LPM19c. The effect of peptides M1–M8 on adhesion was investigated by incubating the indicated peptide with sGP Ib $\alpha$ -coated wells for 30 min at 37°C before the addition of cells. Data are expressed as percent adhesion of control treatment.

**Neutrophil Adhesion to Surface-adherent Platelets.** Neutrophil adhesion to surface-adherent platelets was investigated as previously described (11). The effect of  $\alpha_M$  peptides on leukocyte adhesion to platelets was examined by preincubating surface-adherent platelets with peptide (1–1,000 nM) or vehicle for 30 min at 37°C. Data are expressed as percent adhesion of control treatment.

**Site-directed Mutagenesis, Expression, and Purification of I Domain Fusion Proteins.** The cDNAs of  $\alpha_M$ I domain (675 nucleotides, R<sup>115</sup>–S<sup>340</sup>),  $\alpha_L$ I domain (630 nucleotides, P<sup>120</sup>–S<sup>330</sup>), and mouse  $\alpha_M$ I domain (675 nucleotides, L<sup>115</sup>–S<sup>340</sup>) were cloned and inserted into the pGEX-5X-3 expression vector (22). All wild-type and mutant I domains were expressed as glutathione S-transferase (GST) fusion proteins. Mutations were created in these I domains and intact  $\alpha_L$  by oligonucleotide-directed mutagenesis. The selective introduction of the desired mutations into the I domains was confirmed by DNA sequence analyses. The GST-I domain fusion proteins were purified by adsorption onto glutathione-Sepharose 4B (Amersham Biosciences) and eluted with buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM glutathione, 2.5 mM CaCl<sub>2</sub>. Such preparations of the fusion proteins were ~90% pure as assessed by SDS-PAGE.

**GP Ib $\alpha$  Binding to the I Domains.** To test the interaction of the wild-type  $\alpha_M$ ,  $\alpha_L$ , and chimeric I domains with biotinylated GP Ib $\alpha$ , 96-well plates (Immulon 4BX; Dynex Technologies Inc.) were coated with the I domains at 50  $\mu\text{g/ml}$  and blocked with 2% BSA. sGP Ib $\alpha$  in 20 mM Tris-HCl, pH 7.6, containing 100 mM NaCl and 2 mM CaCl<sub>2</sub>, was added to the wells and incubated for 1 h at 37°C. After washing, bound GP Ib $\alpha$  was detected using avidin-alkaline phosphatase and *p*-nitrophenyl phosphate. Background reaction on BSA-coated wells was subtracted.

**BIAcore Surface Plasmon Resonance Analysis.** Real-time protein-protein interactions were examined using surface plasmon resonance on a BIAcore 1000 (BIAcore AB). Immobilization of peptides was performed via thiol coupling onto a sensor chip C1 using 20 mM Tris, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, pH 7.4, as running buffer at a flow rate of 5  $\mu\text{l min}^{-1}$ . Modified versions of peptides containing an amino-terminal cysteine residue were synthesized and then diluted in 10 nM acetate buffer, pH 5.0 (coupling buffer). The peptide was loaded on the chip for 16 min and immobilization occurred via thiol disulfide exchange. Identical treatment was applied to the reference flow cell without peptide (control surface). For analysis, sGP Ib $\alpha$  diluted into running buffer was injected at a flow rate of 10  $\mu\text{l/min}$ . Binding (resonance unit [RU]) was measured as a function of time(s). Binding data (after subtracting nonspecific background binding to the control surface) are presented as sensorgrams, binding curves, and Scatchard plots (31).

**In Vitro Analysis of Cellular Adhesion under Laminar Flow Conditions.** The laminar flow chamber used in this assay has been described (32). 25 mm<sup>2</sup> diameter glass coverslips (Assistant; Carolina Biological Supply Company) were coated with a solution of 20  $\mu\text{g/ml}$  soluble P-selectin (provided by R. Camphausen, Genetics

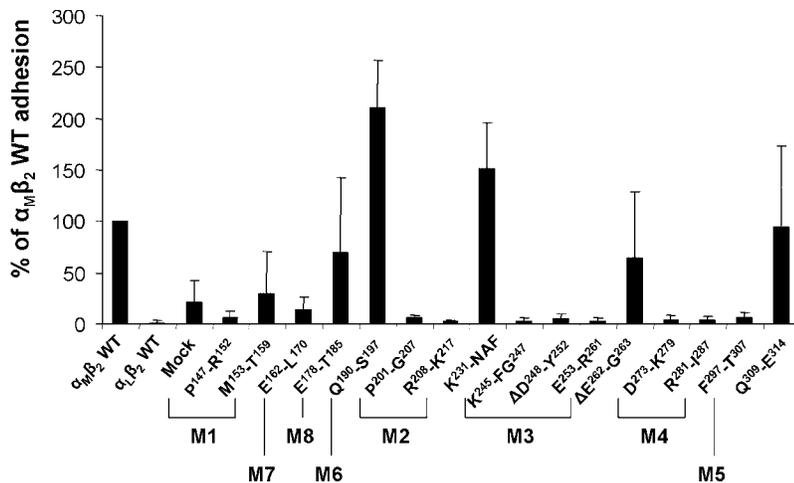
Institute, Cambridge, MA) and 40  $\mu\text{g/ml}$  sGP Ib $\alpha$  for 2 h at room temperature. Nonspecific interactions were blocked with PBS containing 1% human serum albumin for 1 h at room temperature and with 0.1% Tween 20 immediately before use. The effect of M2 and sCM2 peptides on THP-1 cell rolling and firm arrest was examined by incubating 50  $\mu\text{M}$  of the peptide with the ligand-coated glass coverslip for 1 h at room temperature. To block PSGL-1 and  $\alpha_M\beta_2$  interactions, THP-1 cells were incubated with 20  $\mu\text{g/ml}$  KPL-1 (BD Biosciences) or 20  $\mu\text{g/ml}$  LPM19c mAb, respectively, for 20 min before perfusion.  $\alpha_M\beta_2$  was activated by treating cells with 10  $\mu\text{g/ml}$  KIM127 for 5 min before perfusion. The rolling and firm adhesive events scored were ligand specific as confirmed in parallel determinations on control substrates coated with human serum albumin. Coverslips were inserted in the flow chamber and  $0.6 \times 10^6/\text{ml}$  THP-1 cells were drawn across at an estimated shear stress of 0.75 dynes/cm<sup>2</sup> using a syringe pump (Harvard Apparatus). After 3 min of perfusion, the number of rolling and arrested cells was quantified in each of five random 10x fields (area ~0.3 mm<sup>2</sup>) by an investigator blinded to treatment.

**Statistics.** Data are presented as the mean  $\pm$  SD or SEM. Groups were compared using the nonpaired *t* test. Rolling and arrest data were analyzed using repeated measure ANOVA with a Bonferroni corrective for multiple comparisons. *P* values <0.05 were considered significant.

## Results

**Binding of GP Ib $\alpha$  to Mutant Cell Lines.** Our previous report indicated that the  $\alpha_M$ I domain serves as a recognition site for GP Ib $\alpha$  (11). The adhesion of  $\alpha_M\beta_2$ -bearing cells to sGP Ib $\alpha$  was inhibited by LPM19c, an mAb that binds to the  $\alpha_M$ I domain. To establish definitively that the  $\alpha_M$ I domain serves as a recognition site for GP Ib $\alpha$ , we transfected 293 cells with wild-type  $\alpha_M\beta_2$ ,  $\alpha_L\beta_2$ , or a chimeric  $\alpha_L\beta_2$  receptor that contained the I domain of  $\alpha_M\beta_2$  (i.e.,  $\alpha_L(\alpha_M)\beta_2$ -transfected 293 cells).  $\alpha_L\beta_2$ -transfected 293 cells did not adhere to sGP Ib $\alpha$ . In contrast,  $\alpha_L(\alpha_M)\beta_2$ -transfected 293 cells adhered robustly to sGP Ib $\alpha$  in a manner similar to  $\alpha_M\beta_2$ -transfected 293 cells, indicating that the  $\alpha_M$ I domain is required for adhesion to sGP Ib $\alpha$ . These data suggest that although  $\alpha_M\beta_2$  and  $\alpha_L\beta_2$  are highly homologous integrins, the lack of binding of sGP Ib $\alpha$  to  $\alpha_L\beta_2$  might be the result of sequence and/or structural differences.

As the first step to define the binding site for GP Ib $\alpha$  within the  $\alpha_M$ I domain, mutant cell lines, each expressing a mutant  $\alpha_M\beta_2$  in which a short  $\alpha_M$ I domain sequence, corresponding to a structural unit in the crystal structure (8, 9), was replaced for the corresponding region of the  $\alpha_L$ I domain, were tested for their adhesion to immobilized sGP Ib $\alpha$ . 16 segments were switched from their original  $\alpha_M$ I domain to their counterparts in  $\alpha_L$  (16, 20, 23). These segment swaps are placed throughout the P<sup>147</sup>–Q<sup>314</sup> region of the  $\alpha_M$ I domain, D<sup>132</sup>–A<sup>318</sup>, and are intended to cover its entire hydrated surface (8, 9). For such experiments to be readily interpretable, cell lines expressing high and similar levels of wild-type and mutant receptors were selected by cell sorting using mAbs OKM1 and IB4 followed by cloning by limiting dilution. The expression of the receptors as assessed by the mean fluorescence intensity (MFI) in



**Figure 1.** Binding of GP Ib $\alpha$  to mutant cell lines. Adhesion of  $10^5$   $\alpha_M\beta_2^-$ ,  $\alpha_I\beta_2^-$ , and mutant  $\alpha_M\beta_2^-$ -expressing 293 cells to sGP Ib $\alpha$ -coated wells. Adhesion was promoted by the addition of 5  $\mu$ g/ml of the stimulating mAb KIM 127 and quantified by measuring the fluorescence of BCECF AM-loaded cells. Data are expressed as percent wild-type  $\alpha_M\beta_2$  adhesion (mean  $\pm$  SD of three to five independent experiments). A series of peptides (M1–M8) corresponding to the wild-type  $\alpha_M$ I domain sequences were synthesized and tested for their ability to interact with sGP Ib $\alpha$  (Table I). Because the sequences within several of the negative mutants were contiguous, linear peptides spanning these sequences were synthesized: P<sup>147</sup>–T<sup>159</sup> (M1), P<sup>201</sup>–K<sup>217</sup> (M2), K<sup>245</sup>–R<sup>261</sup> (M3), D<sup>273</sup>–I<sup>287</sup> (M4), F<sup>297</sup>–T<sup>307</sup> (M5), Q<sup>190</sup>–S<sup>197</sup> (M6), E<sup>162</sup>–L<sup>170</sup> (M7), and E<sup>178</sup>–T<sup>185</sup> (M8). All peptides correspond to negative mutant sequences except M6 and M8, which correspond to intermediate and positive mutant sequences, respectively.

FACS<sup>®</sup> analyses differed by less than twofold from the internal wild-type control (not depicted).

The results of adhesion of 16 mutants to sGP Ib $\alpha$  are summarized in Fig. 1. The data are expressed as the percent adhesion of the wild-type  $\alpha_M\beta_2$ -expressing cells to sGP Ib $\alpha$ . Substitutions for the following regions of  $\alpha_M$ I domain abrogated adhesion:  $\alpha_M$ (P<sup>147</sup>–R<sup>152</sup>),  $\alpha_M$ (M<sup>153</sup>–T<sup>159</sup>),  $\alpha_M$ (E<sup>162</sup>–L<sup>170</sup>),  $\alpha_M$ (P<sup>201</sup>–G<sup>207</sup>),  $\alpha_M$ (R<sup>208</sup>–K<sup>217</sup>),  $\alpha_M$ (K<sup>245</sup>–F<sup>247</sup>),  $\alpha_M$ ( $\Delta$ D<sup>248</sup>–Y<sup>252</sup>),  $\alpha_M$ (E<sup>253</sup>–R<sup>261</sup>),  $\alpha_M$ (D<sup>273</sup>–K<sup>279</sup>),  $\alpha_M$ (R<sup>281</sup>–I<sup>287</sup>), and  $\alpha_M$ (F<sup>297</sup>–T<sup>307</sup>). These cell lines form a group of negative mutants. As the essential control, the  $\alpha_I\beta_2^-$ -expressing cells adhered poorly to sGP Ib $\alpha$ , consistent with lack of interaction of  $\alpha_I\beta_2$  with sGP Ib $\alpha$ .

The lack of adhesion of these mutants was not the result of decreased surface expression of the receptor because there was no correlation between the level of adhesion and expression. Specifically, surface expression of the  $\alpha_M$ (P<sup>201</sup>–G<sup>207</sup>) mutant was 1.5-fold higher than that of the cells expressing the wild-type  $\alpha_M\beta_2$  cells, but adhesion was abrogated completely. Surface expression of negative mutants,  $\alpha_M$ (P<sup>147</sup>–R<sup>152</sup>),  $\alpha_M$ (R<sup>208</sup>–K<sup>217</sup>),  $\alpha_M$ (E<sup>253</sup>–R<sup>261</sup>), and  $\alpha_M$ (D<sup>273</sup>–K<sup>279</sup>) was very similar to that of the wild-type receptor. Adhesion of  $\alpha_M$ (E<sup>178</sup>–T<sup>185</sup>) and  $\Delta$ E<sup>262</sup>G<sup>263</sup> was partially affected. The maximal level of adhesion to sGP Ib $\alpha$  reached 69 and 64%, respectively, of wild-type  $\alpha_M\beta_2$  cells. These two receptors were classified as intermediate mutants.

The following mutants supported the same or higher levels of adhesion to sGP Ib $\alpha$  compared with the cells expressing the wild-type  $\alpha_M\beta_2$  receptor:  $\alpha_M$ (Q<sup>190</sup>–S<sup>197</sup>),  $\alpha_M$ (K<sup>231</sup>–NAF<sup>234</sup>),  $\alpha_M$ (E<sup>262</sup>–G<sup>263</sup>), and  $\alpha_M$ (Q<sup>309</sup>–E<sup>314</sup>). The cell lines exhibiting adhesion similar to or greater than the wild-type  $\alpha_M\beta_2$  cells were identified as positive mutants. Two of these positive mutants,  $\alpha_M$ (Q<sup>190</sup>–S<sup>197</sup>) and  $\alpha_M$ (K<sup>231</sup>–NAF<sup>234</sup>), bound sGP Ib $\alpha$  better than wild-type  $\alpha_M\beta_2$ . Previous analyses using these mutants showed that they bound neither an  $\alpha_M\beta_2$  activation-independent ligand, NIF (20), nor  $\alpha_M\beta_2$  activation-dependent ligands, C3bi (17) and fibrinogen (23), to a greater extent than wild-type receptor. Thus, the enhanced binding of these mutants appears to be selective for sGP Ib $\alpha$  recognition. In further

studies, we found that binding of the  $\beta_2$  activation-specific reporter mAb 24 was similar for wild-type and the  $\alpha_M$ (Q<sup>190</sup>–S<sup>197</sup>) and  $\alpha_M$ (K<sup>231</sup>–NAF<sup>234</sup>) mutants. In contrast, increased binding of the  $\alpha_M$  activation-specific reporter CBRM1/5 to  $\alpha_M$ (K<sup>231</sup>–NAF<sup>234</sup>) compared with wild-type  $\alpha_M\beta_2$  and  $\alpha_M$ (Q<sup>190</sup>–S<sup>197</sup>) suggests possible conformation change of the  $\alpha_M$ (K<sup>231</sup>–NAF<sup>234</sup>) mutant (not depicted). Together, these observations suggest that the activation states of  $\alpha_M\beta_2$  may reflect the reporter ligand or mAb used, and these two mutants might be functionally activated with respect to sGP Ib $\alpha$  recognition.

**Interaction of GP Ib $\alpha$  with the  $\alpha_M$ I Domain Peptides.** In subsequent analyses, we focused on the 11 negative mutants. A series of peptides (M1–M8) corresponding to the wild-type  $\alpha_M$ I domain sequences were synthesized and tested for their ability to interact with sGP Ib $\alpha$ . Because the sequences within several of the negative mutants were contiguous, the following linear peptides spanning these sequences were synthesized: P<sup>147</sup>–T<sup>159</sup> (designated M1), P<sup>201</sup>–K<sup>217</sup> (M2), K<sup>245</sup>–R<sup>261</sup> (M3), D<sup>273</sup>–I<sup>287</sup> (M4), F<sup>297</sup>–T<sup>307</sup> (M5), Q<sup>190</sup>–S<sup>197</sup> (M6), E<sup>162</sup>–L<sup>170</sup> (M7), and E<sup>178</sup>–T<sup>185</sup> (M8). All peptides correspond to negative mutant sequences except M6 and M8, which correspond to intermediate and positive mutant sequences, respectively.

Next, we examined the effect of these  $\alpha_M$ I domain peptides on  $\alpha_M\beta_2$ -dependent adhesion to sGP Ib $\alpha$  or fibrinogen (Table I). 50  $\mu$ M peptides were preincubated in ligand-coated wells,  $\alpha_M\beta_2$ -expressing 293 cells were added, and adhesion was stimulated with KIM 127. Mac-1 293 cells adhered to sGP Ib $\alpha$  and fibrinogen, and this adhesion was blocked by the anti-CD11b mAb LPM19c, indicating that adhesion is predominantly  $\alpha_M\beta_2$  dependent (Table I). Peptide M2 inhibited adhesion to sGP Ib $\alpha$  (percent inhibition =  $74 \pm 13$ ;  $P < 0.01$ ). Interestingly, M2 had no effect on cellular adhesion to fibrinogen. All other peptides had minimal or no effect on  $\alpha_M\beta_2$ -expressing 293 cell adhesion to sGP Ib $\alpha$  or fibrinogen. M2, but not a scrambled version (scM2), blocked  $\alpha_M\beta_2$ -dependent adhesion to sGP Ib $\alpha$  in a dose-dependent manner ( $IC_{50} = 20 \mu$ M; Fig. 2). Taken together, these observations suggest that the  $\alpha_M$ I domain se-

**Table I.** Effect of  $\alpha_M I$  Domain Peptides on  $\alpha_M \beta_2$  293 Cell Adhesion to sGP Ib $\alpha$  and Fibrinogen

Peptide or mAb	$\alpha_M I$ domain sequence	Peptide sequence	Percent $\alpha_M \beta_2$ 293 adhesion to sGP Ib $\alpha$	Percent $\alpha_M \beta_2$ 293 adhesion to fibrinogen
M1	P <sup>147</sup> -T <sup>159</sup>	PHDFRRMKEFVST	76 ± 18	118 ± 27
M2	P <sup>201</sup> -K <sup>217</sup>	PITQLLGRTHATGIRK	26 ± 13 <sup>a</sup>	102 ± 46
M3	K <sup>245</sup> -R <sup>261</sup>	KFGDPLGYEDVIPEADR	94 ± 20	136 ± 32
M4	D <sup>273</sup> -I <sup>287</sup>	DAFRSEKSRQELNTI	110 ± 36	115 ± 8
M5	F <sup>297</sup> -T <sup>307</sup>	FQVNNFEALKT	96 ± 16	105 ± 12
M6	Q <sup>190</sup> -S <sup>197</sup>	QNNPNPRS	93 ± 17	107 ± 3
M7	E <sup>162</sup> -L <sup>170</sup>	EQLKKSCTL	79 ± 31	103 ± 3
M8	E <sup>178</sup> -T <sup>185</sup>	EEFRIHFT	66 ± 27	104 ± 11
C-M2	P <sup>201</sup> -K <sup>217</sup>	CPITQLLGRTHATGIRK	26 ± 1 <sup>a</sup>	ND
scM2		LGTRITHQRTGPTIKL	80 ± 15	ND
LPM19c	Anti- $\alpha_M I$ domain		6.3 ± 5.7 <sup>b</sup>	6.1 ± 6.6 <sup>b</sup>

The adhesion of  $\alpha_M \beta_2$ -expressing 293 cells to sGP Ib $\alpha$ - or fibrinogen-coated microtiter wells was stimulated by the addition of 5  $\mu$ g/ml KIM 127 in the presence and absence of 50  $\mu$ M  $\alpha_M I$  domain peptides or 10  $\mu$ g/ml antibody (LPM19c). Data are expressed as percent adhesion with vehicle alone. Mean  $\pm$  SD,  $n = 3-5$ .

<sup>a</sup>P < 0.01.

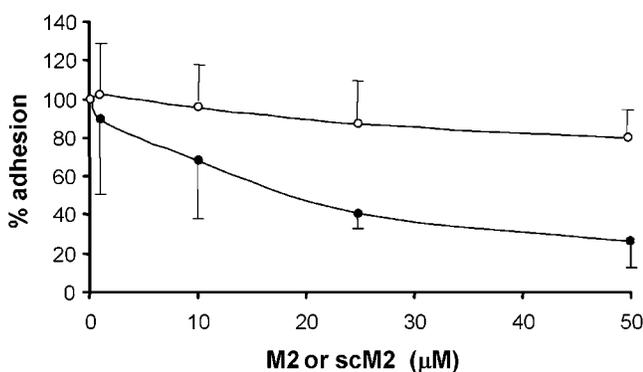
<sup>b</sup>P < 0.0001.

quence corresponding to M2 (P<sup>201</sup>-K<sup>217</sup>), which spans an exposed loop and amphipathic  $\alpha 4$  helix in the three-dimensional structure of the  $\alpha_M I$  domain, contributes to  $\alpha_M \beta_2$  binding of GP Ib $\alpha$ .

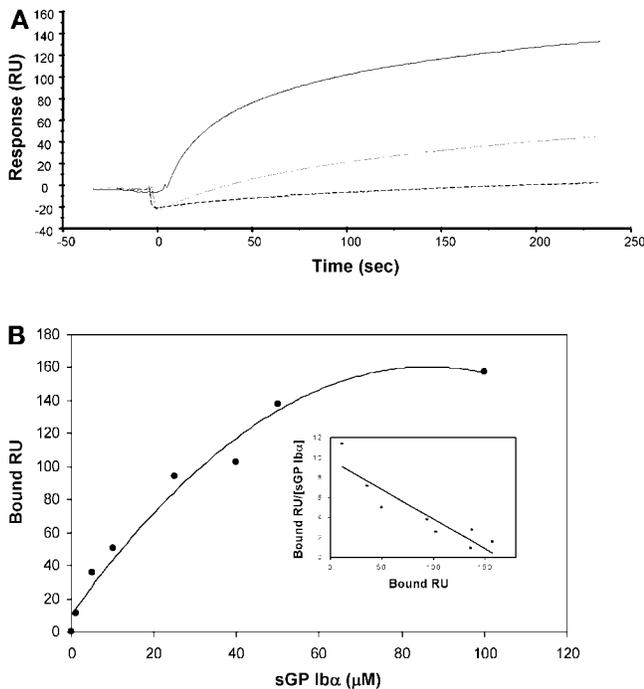
*Real-Time Detection of Protein-Protein Interaction by BIAcore Analyses Reveals Specific, Direct Binding of GP Ib $\alpha$  to M2 Peptide.* To examine direct interactions between GP Ib $\alpha$  and M2, we used an in vitro real-time binding assay using surface plasmon resonance (BIAcore). M2 and scM2 peptides were modified by addition of an amino-terminal cysteine and immobilized via thiol coupling on a C1 sensor chip. Cys-M2, but not Cys-scM2, inhibited  $\alpha_M \beta_2$ -dependent adhesion to sGP Ib $\alpha$ , verifying that cysteine addition did not adversely

affect peptide structure (Table I). Increasing concentrations of purified sGP Ib $\alpha$  in running buffer containing 2 mM MgCl<sub>2</sub> were injected over the chip surface and the sensorgrams were recorded. As shown in Fig. 3, the sensorgrams detected little interaction between sGP Ib $\alpha$  and control chips or chips containing immobilized scM2. Significant interaction responses were detected for sGP Ib $\alpha$  with M2. Apparent equilibrium dissociation constant was estimated from the equilibrium resonance signal as a function of analyte (GP Ib $\alpha$ ) concentration (kD  $\sim$ 20  $\mu$ M) and calculated by Scatchard analysis (kD = 17  $\mu$ M). Similar results were obtained when purified sGP Ib $\alpha$  in running buffer containing 2 mM CaCl<sub>2</sub> were injected over the chip surface (not depicted). Thus, BIAcore analysis revealed real-time, direct binding for bimolecular interactions between GP Ib $\alpha$  and M2.

*GP Ib $\alpha$  Binding to "Triple Mutants" of the  $\alpha_M I$  Domain.* To begin localization of specific amino acid residues within the  $\alpha_M I$  domain involved in GP Ib $\alpha$  recognition, recombinant fragments containing wild-type  $\alpha_M I$  domain, R<sup>115</sup>-S<sup>340</sup>, wild-type  $\alpha_L I$  domain, P<sup>120</sup>-S<sup>330</sup>, and a mutant  $\alpha_M I$  domain were expressed as GST fusion proteins in *Escherichia coli*. The mutant  $\alpha_M I$  domain contained a swap of the P<sup>201</sup>-K<sup>217</sup> segment, which we implicated in sGP Ib $\alpha$  binding to the corresponding residues of the  $\alpha_L I$  domain. The recombinant proteins were purified from the bacterial lysates on glutathione-Sepharose. A facile binding assay for quantifying sGP Ib $\alpha$  binding to the recombinant I domains was developed by measuring the binding of biotinylated sGP Ib $\alpha$  to the recombinant I domains immobilized onto 96-well plastic plates. As shown in Fig. 4, GP Ib $\alpha$  exhibited minimal reaction with  $\alpha_L I$  domain. Binding was observed and was similar with both human and mouse  $\alpha_M I$  domains. With the  $\alpha_M I$  domains, binding was dependent on the sGP Ib $\alpha$  con-



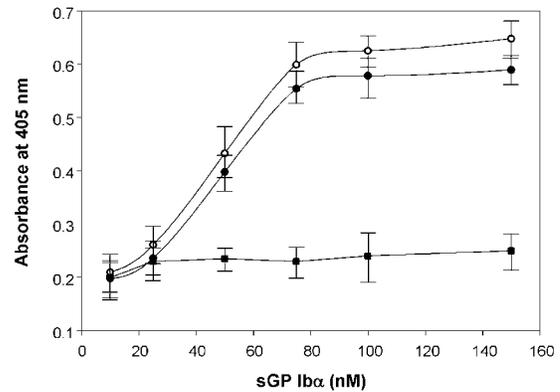
**Figure 2.** Effect of M2 peptide on  $\alpha_M \beta_2$ -dependent adhesion to GP Ib $\alpha$ . 293 cells expressing  $\alpha_M \beta_2$  were added to sGP Ib $\alpha$ -coated wells preincubated with 0–50  $\mu$ M M2 (●) or scM2 (○) peptides. Adhesion was promoted by the addition of 5  $\mu$ g/ml of the stimulating mAb KIM 127 and quantified by measuring the fluorescence of BCECF AM-loaded cells. Data represent mean  $\pm$  SD,  $n = 3$  independent experiments.



**Figure 3.** BIAcore analysis of the interaction between GP Iba and M2. (A) 0–100  $\mu\text{M}$  sGP Iba in running buffer injected over control chips (dashed line) or chips immobilized with Cys-M2 (solid line) or Cys-scM2 (dotted line) for 4 min at a flow rate of 10  $\mu\text{l}/\text{min}$  followed by running buffer alone. In all cases, sensograms obtained with control flow cells were subtracted from those obtained with immobilized peptides. (B) Plot of RU as a function of GP Iba concentration. The inset shows Scatchard analysis to obtain apparent equilibrium dissociation constant ( $K_D = 17 \mu\text{M}$ ). RU, resonance unit.

centration with 50% maximal binding observed at 50 nM sGP Iba, which was used in subsequent experiments. As shown in Fig. 5 A, the interaction of the  $\alpha_M\text{I}$  domain “swap” mutant with biotinylated sGP Iba was greatly diminished compared with wild-type  $\alpha_M\text{I}$  domain and similar to that of the  $\alpha_L\text{I}$  domain. Essentially, no specific binding of sGP Iba to this mutant I domain was detected. This result is consistent with our BIAcore experiments showing a direct interaction between sGP Iba and immobilized M2 peptide corresponding to this P<sup>201</sup>–K<sup>217</sup> segment.

To begin to identify the individual residues within the P<sup>201</sup>–K<sup>217</sup> segment that mediated sGP Iba recognition, a series of six triple mutants were created. In each of these triple mutants, a set of three consecutive amino acids within the  $\alpha_M\text{I}$  domain was changed to the corresponding  $\alpha_L$  residues. If the  $\alpha_M$  and  $\alpha_L$  residues were the same, the amino acid was mutated to alanine. After the DNA sequence of each mutant I domain was confirmed, it was expressed in *E. coli* and purified on glutathione-Sepharose. When analyzed by SDS-PAGE, each mutant migrated as a single band of  $\sim 52$  kD (not depicted). sGP Iba binding to each triple mutant was then assessed. The results in Fig. 5 A show the binding of each of the six triple mutants to 50 nM biotinylated sGP Iba. Of the six triple mutants, three, H<sup>210</sup>–A<sup>212</sup>, T<sup>213</sup>–I<sup>215</sup>, and R<sup>216</sup>–K<sup>217</sup>, showed a significant

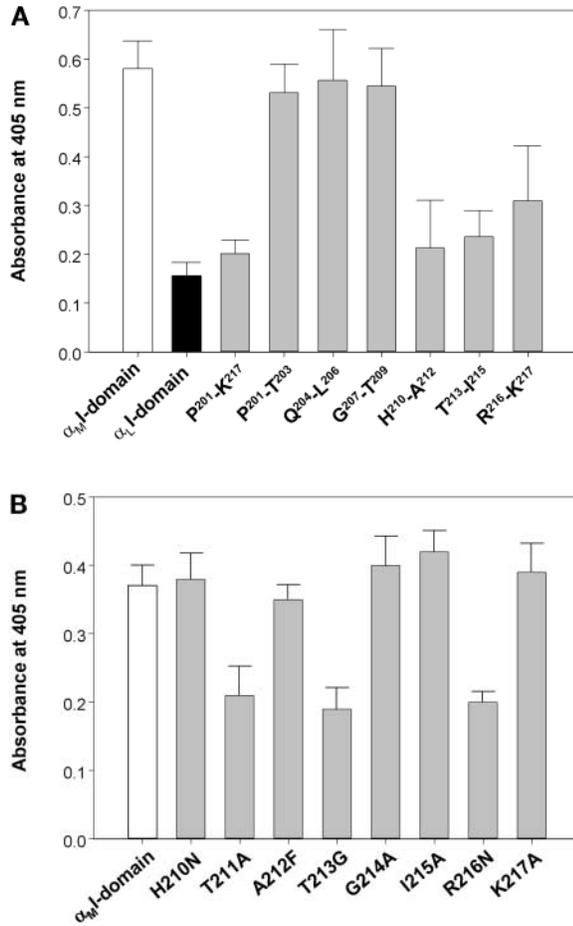


**Figure 4.** GP Iba binding to recombinant I domains. Biotinylated sGP Iba was added at the indicated concentrations to  $\alpha_M\text{I}$  domain GST fusion proteins immobilized on microtiter wells. The various I domains are: human  $\alpha_M\text{I}$  domain ( $\circ$ ), mouse  $\alpha_M\text{I}$  domain ( $\bullet$ ), and the  $\alpha_L\text{I}$  domain ( $\blacksquare$ ). Incubations were performed in the presence of 2 mM  $\text{Ca}^{2+}$ , and samples were processed as indicated in Materials and Methods. Each dataset is the mean  $\pm$  SEM of at least three independent experiments.

reduction in sGP Iba binding. Three mutants, P<sup>201</sup>–T<sup>203</sup>, Q<sup>204</sup>–L<sup>206</sup>, and G<sup>207</sup>–T<sup>209</sup>, did not impair binding to GP Iba. These results, taken in light of the observation that mutating the entire region spanning P<sup>201</sup>–G<sup>207</sup> abolished cell adhesion to sGP Iba (Fig. 1), suggest that the inactivation of sGP Iba binding requires alteration of more than one residue within the P<sup>201</sup>–G<sup>207</sup> (i.e., no single residue within the triple mutants, P<sup>201</sup>–T<sup>203</sup>, Q<sup>204</sup>–L<sup>206</sup>, and G<sup>207</sup>–T<sup>209</sup>, decreases affinity detectably), or the conformation of the P<sup>201</sup>–G<sup>207</sup> segment, which corresponds to a portion of a loop within the  $\alpha_M\text{I}$  domain, is necessary for binding, and, again, no single substitution alters the conformation of this region sufficiently to prevent binding.

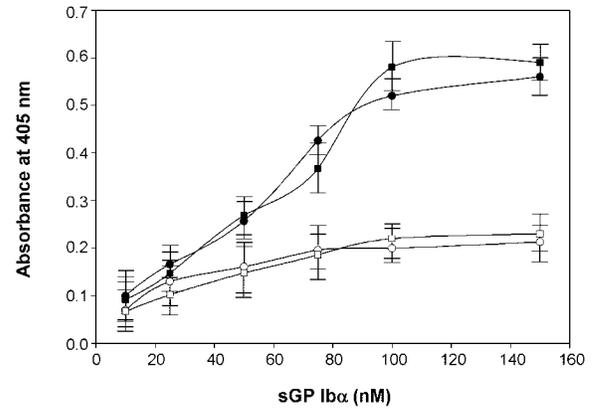
**GP Iba Binding to “Single Point Mutants” of the  $\alpha_M\text{I}$  Domain.** Next, within the three triple mutants with reduced sGP Iba binding, each of the three amino acids was mutated individually to the corresponding residue in  $\alpha_L\text{I}$  domain or in case of identical residues in two I domains, the amino acid was replaced with an alanine. After confirming the DNA sequences of these single mutants, each of the GST fusion proteins was purified. The mutant fusion protein carrying the K<sup>217</sup>Y substitution was insoluble. Therefore, K<sup>217</sup> was mutated to Ala, and this  $\alpha_M\text{I}$  domain with a K<sup>217</sup>A substitution was readily purified, yielding a total of eight single mutants. The capacity of the eight single mutants to bind sGP Iba is summarized in Fig. 5 B. Within each of the three triple mutants with reduced sGP Iba recognition, only one of the three single mutants exhibited reduced sGP Iba binding. These three single mutants showing reduced binding were T<sup>211</sup>A, T<sup>213</sup>G, and R<sup>216</sup>N.

**Development of a Chimeric  $\alpha_L\text{I}$  Domain with GP Iba Binding Activity.** Loss of GP Iba binding function in these single mutants could reflect direct involvement of the specific residues in GP Iba binding or conformational perturbation of the resulting  $\alpha_M\text{I}$  domain due to substitutions at these positions. A gain in function approach was used to distinguish between these possibilities by introducing the identi-



**Figure 5.** GP Iba binding to  $\alpha_M$ I domain triple mutants (A) and  $\alpha_M$ I domain single mutants (B). The GST fusion proteins were adsorbed onto wells of microtiter plates, and sGP Iba binding was measured using a 50-nM concentration of ligand. Each dataset is the mean  $\pm$  SEM of at least three independent experiments.

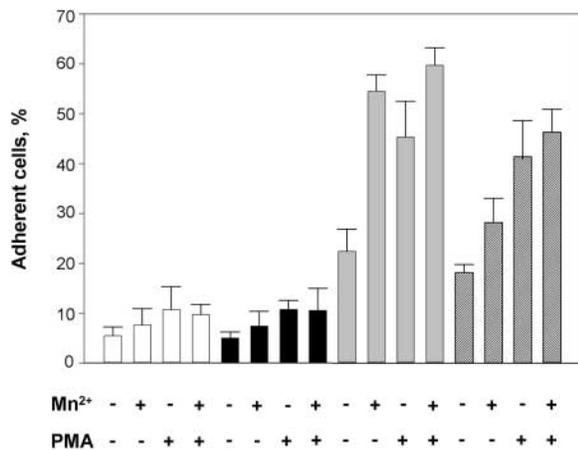
fied point mutations into the  $\alpha_L$ I domain. T<sup>211</sup> is conserved in both  $\alpha_M$ I and  $\alpha_L$ I domains. Therefore, chimeric I domains containing either a single G<sup>213</sup>T substitution or two substitutions, G<sup>213</sup>T and N<sup>216</sup>R, in the  $\alpha_L$ I domain backbone were created. These mutant  $\alpha_L$ I domains were expressed as GST fusion proteins, purified, and their sGP Iba binding properties were evaluated. The chimeric I domain harboring both the G<sup>213</sup>T and N<sup>216</sup>R mutations bound sGP Iba with an affinity substantially greater than wild-type  $\alpha_L$ I domain and the I domain containing the single G<sup>213</sup>T mutation (Fig. 6). Indeed, the binding capability of double substituted chimeric I domain was comparable to the wild-type  $\alpha_M$ I domain. To confirm that these two amino acid residues are sufficient to impart sGP Iba recognition to the mutated  $\alpha_L$ I domain, we performed additional BIAcore binding assays with immobilized  $\alpha_L$ I domain peptide (C<sup>201</sup>HVKHMLLTNTFGAIN<sup>Y217</sup>, termed C-L2) corresponding to the P<sup>201</sup>-K<sup>217</sup> sequence within  $\alpha_M$ I domain and the double substituted mutant C<sup>201</sup>HVKHMLLTN-TFTAIR<sup>Y217</sup> (C-muL2). Binding assays were also performed with a mutant M2 peptide containing T<sup>211</sup>A,



**Figure 6.** GP Iba binding to the chimeric  $\alpha_L$ I domain. Biotinylated sGP Iba binding was measured as in Fig. 1 to wild-type  $\alpha_M$ I domain (●), the  $\alpha_L$ I domain (□), the chimeric  $\alpha_L$ I domain (○) containing the single G(213)T mutation, and the chimeric  $\alpha_L$ I domain containing the double G<sup>213</sup>T and N<sup>216</sup>R substitutions (■). Values are presented as the mean  $\pm$  SEM of at least three separate experiments.

T<sup>213</sup>G, and R<sup>216</sup>N substitutions (C<sup>201</sup>PITQLLGRTH-AAGGINK<sup>217</sup>, termed C-muM2) corresponding to the three single mutants showing reduced binding in the purified mutant I domain binding assays (Fig. 5 B). The sensorgrams detected little interaction between 50  $\mu$ M sGP Iba and chips containing immobilized C-L2 (RU = 10) or C-muM2 (RU = 25). Significant interaction responses were detected for sGP Iba with C-muL2 (RU = 224).

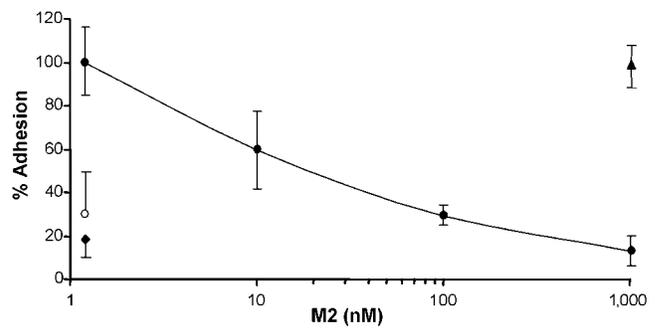
*Role of the Identified Amino Acids in the Context of Intact  $\alpha_M\beta_2$ .* The role of T<sup>213</sup> and R<sup>216</sup> in the GP Iba binding function of the intact receptor was investigated. The T<sup>213</sup>G and R<sup>216</sup>N substitutions were introduced into the cDNA for the  $\alpha_L$  subunit using site-directed mutagenesis and coexpressed with the cDNA for the  $\beta_2$  subunit in 293 cells. Wild-type  $\alpha_L\beta_2$  and  $\alpha_M\beta_2$  were also transiently expressed in these cells as controls. 48 h after transfection, the cells were detached from tissue culture plates, and receptor expression levels were evaluated by FACS<sup>®</sup>.  $\alpha_M$  expression was evaluated with OKM1,  $\beta_2$  expression with IB4, and  $\alpha_L$  expression with TS1/22. The expression levels of both the  $\alpha$  and  $\beta$  subunits of the integrins were comparable (not depicted). Next, the function of the receptors was assessed by evaluating their adhesion to immobilized sGP Iba. PMA, Mn<sup>2+</sup>, or their combination were used to activate the integrins on the cells. As shown in Fig. 7, mock-transfected cells or cells expressing wild-type  $\alpha_L\beta_2$  showed little adhesion to sGP Iba under all conditions. When  $\alpha_M\beta_2$ -expressing cells were stimulated with PMA, Mn<sup>2+</sup>, or their combination, their adhesion to sGP Iba increased markedly compared with the nonstimulated cells. The chimeric  $\alpha_L\beta_2$  cells adhered to sGP Iba considerably better than the  $\alpha_L\beta_2$  cells or mock-transfected cells. The adhesion of the chimeric  $\alpha_L\beta_2$  cells approached that of the wild-type  $\alpha_M\beta_2$  cells. We considered whether inserting these amino acid residues into  $\alpha_L$  might affect the activation state of chimeric  $\alpha_L\beta_2$  such that the increase in binding might reflect allosteric changes in the inte-



**Figure 7.** GP Ib $\alpha$  binding to the chimeric  $\alpha_L\beta_2$ . Adhesion of mock-transfected 293 cells (open bars),  $\alpha_L\beta_2$ -transfected cells (solid bars),  $\alpha_M\beta_2$ -transfected cells (gray bars), and cells expressing the chimeric  $\alpha_L\beta_2$  receptor (striped bars) to sGP Ib $\alpha$ -coated wells. The percentage of input cells adherent after washing was calculated. Values are the mean  $\pm$  SEM of three individual experiments.

grin. Accordingly, we assessed the activation states of the chimeric integrin using MEM-83, an mAb that reacts with activated  $\alpha_L\beta_2$  (33). In FACS<sup>®</sup> analyses, MEM-83 failed to react with either the  $\alpha_L\beta_2$  or the chimeric  $\alpha_L\beta_2$  transfectants in the unstimulated state (MFI = 14 and 10, respectively), but reacted well and equivalently with both transfectants upon stimulation with the combination of Mn<sup>2+</sup> and PMA (MFI = 79 and 83, respectively). Thus, the activation states of both the  $\alpha_L\beta_2$  and chimeric  $\alpha_L\beta_2$  transfectants, with and without stimulation, were similar.

**$\alpha_M\beta_2$  and GP Ib $\alpha$  Facilitate the Interaction between Leukocytes and Platelets.** Having previously reported that  $\alpha_M\beta_2$  and GP Ib $\alpha$  facilitate the heterotypic interaction between leukocytes and platelets, we turned to examining the effect of M2 on neutrophil adhesion to platelets. Thioglycollate-elicited neutrophils were added to wells containing surface adherent platelets and adhesion was stimulated by the addition of PMA to each well. We verified by FACS<sup>®</sup> analysis that such PMA treatment up-regulated P-selectin expression 29.9-fold in platelets purified and prepared with PGE1 as described in Materials and Methods. Wild-type (Mac-1<sup>+/+</sup>) neutrophils bound to adherent platelets (Fig. 8). In contrast, Mac-1-deficient (Mac-1<sup>-/-</sup>) neutrophils demonstrated markedly reduced adhesion to platelets (percent wild-type adhesion =  $18.2 \pm 8.0$ ) and adhesion of wild-type neutrophils was blocked by the rat anti-mouse  $\alpha_M\beta_2$  mAb M1/70 (percent wild-type adhesion =  $29.9 \pm 19.8$ ), indicating that under these conditions, neutrophil adhesion to platelets is largely  $\alpha_M\beta_2$  dependent, although a complementary role for P-selectin/PSGL-1 is likely operative. M2, but not a scrambled version, inhibited dose dependently (IC<sub>50</sub> = 30 nM) wild-type neutrophil adhesion to platelets. Taken together, these observations indicate that under these experimental conditions, neutrophil adhesion to platelets is mediated primarily by the  $\alpha_M$ I domain sequence P<sup>201</sup>-K<sup>217</sup>.

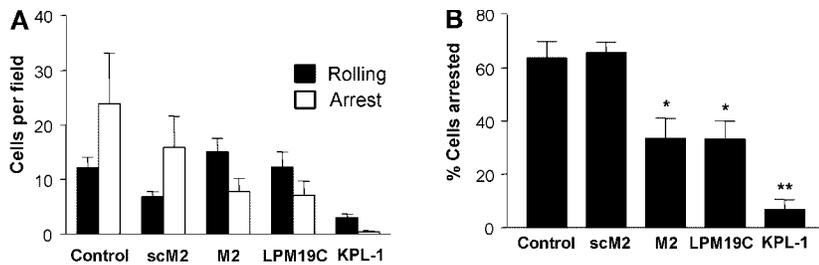


**Figure 8.** Neutrophil binding to platelets is inhibited by M2 peptide. Neutrophils from wild-type (Mac-1<sup>+/+</sup>) or Mac-1-deficient (Mac-1<sup>-/-</sup>, ◆) mice were added to surface-adherent platelets. Adhesion was promoted by the addition of 17 ng/ml PMA. The contribution of  $\alpha_M\beta_2$  to wild-type neutrophil adhesion to platelets was also assayed by the addition of 10  $\mu$ g/ml rat anti-mouse  $\alpha_M$  mAb M1/70 (○). The effect of 0–1,000 nM M2 (●) and scM2 (▲) on neutrophil adhesion was also examined. Adhesion was quantified by measuring the fluorescence of BCECF AM-loaded neutrophils. Data are expressed as percent wild-type neutrophil adhesion with vehicle alone (mean  $\pm$  SD,  $n = 3$  independent experiments).

**M2 Peptide Abrogates the Firm Adhesion of THP-1 Cells under Flow.** To evaluate the potential for M2 to modulate the adhesion of blood cells under flow, we perfused THP-1 cells that express Mac-1 over coverslips coimmobilized with soluble P-selectin and GP Ib $\alpha$  using a parallel plate flow chamber system (0.75 dynes/cm<sup>2</sup>). The number of rolling and arrested cells was quantified on five random fields after 3 min of perfusion. THP-1 cells rolled and arrested on coverslips cocooned with P-selectin and sGP Ib $\alpha$  and the number of rolling (control vs. scM2,  $P > 0.05$ ) or arrested (control vs. scM2,  $P > 0.05$ ) cells was unaffected by scM2 (Fig. 9 A). In contrast, M2 peptide inhibited THP-1 cell arrest, thereby increasing the number of rolling cells visualized. The effect of M2 on cell adhesion was similar to treatment with LPM19c, an anti-CD11b mAb that blocks  $\alpha_M\beta_2$ -dependent binding to sGP Ib $\alpha$ , thereby confirming the involvement of  $\alpha_M\beta_2$  in cell adhesion. Both cell rolling and arrest were abolished by treating cells with KPL-1, an anti-PSGL-1 mAb that blocks P-selectin binding. The effect of M2 on cell arrest was also quantified. Thus, on coverslips incubated with vehicle or scM2, 64 and 66% of THP-1 cells arrested, respectively (Fig. 9 B). After treatment with M2 peptide only 34% of cells arrested ( $P < 0.05$ ). Similarly, after preincubation of THP-1 cells with LPM19C, only 33% of cells arrested ( $P < 0.05$ ). KPL-1 antibody treatment abrogated almost all rolling and subsequent arrest ( $< 10\%$ ;  $P < 0.01$ ).

## Discussion

In this study, we have identified the P<sup>201</sup>-K<sup>217</sup> segment, which spans an exposed loop and amphipathic  $\alpha 4$  helix in the three-dimensional structure of the  $\alpha_M$ I domain, as the binding site for platelet GP Ib $\alpha$ . This conclusion is supported by the following data: (a) mutant cell lines in which the  $\alpha_M$ I domain segments P<sup>201</sup>-G<sup>207</sup> and R<sup>208</sup>-K<sup>217</sup> were



for 20 min before perfusion. The absolute number of rolling and arrested cells was quantified per  $\times 10$  field on five random fields after 3 min of perfusion. Data represent the mean  $\pm$  SEM,  $n = 3$  independent experiments. \*,  $P < 0.05$  versus control peptide; \*\*,  $P < 0.01$  versus control peptide.

**Figure 9.** THP-1 cell adhesion under flow is inhibited by M2 peptide. Soluble GP Ib $\alpha$  was coimmobilized with P-selectin on coverslips.  $0.6 \times 10^6$ /ml THP-1 cells were activated with KIM 127 and drawn across the substrates at 0.75 dynes/cm $^2$  as detailed in Materials and Methods. The effect of M2 and scM2 peptides on THP-1 cell rolling and firm arrest was examined by preincubating 50  $\mu$ M of the peptide with the ligand-coated coverslip. To block PSGL-1 and  $\alpha_M\beta_2$  interactions, THP-1 cells were incubated with 20  $\mu$ g/ml KPL-1 or LPM19c mAb, respectively,

switched to the homologous  $\alpha_L$ I domain segments failed to support adhesion to sGP Ib $\alpha$ , (b) mutation of amino acid residues within P $^{201}$ -K $^{217}$ , H $^{210}$ -A $^{212}$ , T $^{213}$ -I $^{215}$ , and R $^{216}$ -K $^{217}$  resulted in the loss of the binding function of the recombinant  $\alpha_M$ I domains to biotinylated sGP Ib $\alpha$ , (c) synthetic peptides duplicating the P $^{201}$ -K $^{217}$ , but not scrambled versions, directly bound sGP Ib $\alpha$  and inhibited  $\alpha_M\beta_2$ -dependent adhesion to sGP Ib $\alpha$  and adherent platelets, and (d) grafting key amino acids within the P $^{201}$ -K $^{217}$  sequence onto  $\alpha_L$  converted  $\alpha_L\beta_2$  into a GP Ib $\alpha$  binding integrin.

By virtue of binding diverse ligands including, among others, fibrin(ogen) (34, 35), ICAM-1 (36), factor X (37), C3bi (34), high molecular weight kininogen (38), and heparin (4),  $\alpha_M\beta_2$  regulates important leukocyte functions including adhesion, migration, coagulation, proteolysis, phagocytosis, oxidative burst, and signaling (30, 39–42). However, these ligands do not account for all of  $\alpha_M\beta_2$ 's adhesive interactions. Although previous studies have shown that  $\alpha_M\beta_2$  directly facilitates the recruitment of leukocytes at sites of platelet and fibrin deposition (5), the precise platelet counter receptors, including GP Ib $\alpha$  (11) and JAM-3 (43), have been elucidated only recently.

In this study, we have identified key elements of the binding site for GP Ib $\alpha$  within the  $\alpha_M$ I domain. The strategy to define the ligand binding site was based on the difference in the sGP Ib $\alpha$  binding properties of the  $\alpha_M$ I and  $\alpha_L$ I domains and entailed four complementary approaches. In the first approach, a series of homologue-scanning mutants, used previously to map the binding regions for NIF, iC3b, and fibrinogen (16, 20, 23), were screened for adhesion to sGP Ib $\alpha$ . In these mutants, 16 segments at the hydrated surface of the  $\alpha_M$ I domain were replaced with the corresponding segments from the homologous  $\alpha_L$ I domain, which does not bind GP Ib $\alpha$ . 11 mutants lacked the ability to support adhesion sGP Ib $\alpha$ , and alteration of two other regions,  $\alpha_M$ (E $^{178}$ -T $^{185}$ ) and  $\alpha_M$ ( $\Delta$ E $^{262}$ G $^{263}$ ), resulted in the partial loss of adhesive function. Thus, the initial insight provided by these mutant receptors indicated that the sGP Ib $\alpha$  binding interface within the  $\alpha_M$ I domain was composed of several nonlinear sequences.

The second approach entailed the use of synthetic peptides duplicating the sequences of the critical segments in the  $\alpha_M$ I domain. These analyses showed that two critical segments,  $\alpha_M$ (P $^{201}$ -G $^{207}$ ) and  $\alpha_M$ (R $^{208}$ -K $^{217}$ ), may contain amino acid residues that participate directly in binding GP

Ib $\alpha$  because the peptide M2 that spanned P $^{201}$ -K $^{217}$  bound sGP Ib $\alpha$  and inhibited  $\alpha_M\beta_2$ -dependent adhesion to sGP Ib $\alpha$  and adherent platelets. The negative results for other peptides (M1, M3–M5, and M7) synthesized to correspond to other negative mutants (Fig. 1 and Table I) do not exclude a role for other  $\alpha_M$ I domain segments in binding function. These segments may play an accessory role in ligand binding or the short peptides may simply not assume the appropriate conformation for recognition by the ligand. Finally, segments implicated in the binding of sGP Ib $\alpha$  by the negative mutants, other than P $^{201}$ -K $^{217}$ , may reflect interference by  $\alpha_L$ I domain residues rather than residues that actually participate in binding. It is also possible that the activation of  $\alpha_M\beta_2$ , rather than ligand binding per se, was affected adversely by the introduction of these  $\alpha_L$  segments within  $\alpha_M$ I domain. Because these same mutants have been used in previous studies (17, 20, 23) to analyze interaction of other activation-dependent ligands with  $\alpha_M\beta_2$ , such effects on activation would need to specifically perturb GP Ib $\alpha$  recognition.

To obtain direct evidence that the  $\alpha_M$ (P $^{201}$ -K $^{217}$ ) sequence constitutes the functional binding site for the GP Ib $\alpha$ , we turned to a third approach using site-directed mutagenesis of the  $\alpha_M$ I domain. Binding experiments with purified sGP Ib $\alpha$  and GST-I domain mutants provided the independent confirmation that the P $^{201}$ -K $^{217}$  segment is important for sGP Ib $\alpha$  binding because mutations of these residues resulted in significant loss of sGP Ib $\alpha$  binding. These experiments also served to narrow further the binding region to H $^{210}$ -K $^{217}$  and subsequently identified three single mutants showing reduced binding to sGP Ib $\alpha$  (T $^{211}$ A, T $^{213}$ G, and R $^{216}$ N). Of these, T $^{211}$  in  $\alpha_M$ I domain is conserved in the  $\alpha_L$ I domain. Thus, it was the conversion of the residue to A that perturbed GP Ib $\alpha$  binding, suggesting that this substitution exerts a negative influence on the conformation of the  $\alpha_M$ I domain that is required for GP Ib $\alpha$  recognition, rather than participating directly in ligand contact. The positioning of this residue on the interior of the  $\alpha$ 4-helix and facing the central core of the  $\alpha_M$ I domain, according to the crystal structures (8, 9), supports this interpretation. In contrast, T $^{213}$  and R $^{216}$  are appropriately positioned, including when the recent crystal structure of the GP Ib $\alpha$ /vWF A domain (44) is used as a template.

In the fourth approach, the two amino acids (T $^{213}$ G and R $^{216}$ N substitutions) within the  $\alpha_M$ (P $^{201}$ -K $^{217}$ ) were grafted

into the corresponding positions of the  $\alpha_{\text{I}}$  domain in the context of intact  $\alpha_{\text{L}}\beta_2$ . As demonstrated in Figs. 6 and 7, this manipulation imparted GP Ib $\alpha$  binding capacity to the chimeric molecule. Thus, the role of amino acids within the  $\alpha_{\text{M}}(\text{P}^{201}\text{--K}^{217})$  sequence in GP Ib $\alpha$  binding, which initially was inferred from the loss in function experiments, was verified by the gain in function approach. Taken together, the four approaches substantiated independently the role of  $\alpha_{\text{M}}(\text{P}^{201}\text{--K}^{217})$  in GP Ib $\alpha$  binding and provided evidence that two to three critical residues participate in ligand docking.

To date, several lines of evidence have emphasized that multiple ligands share overlapping binding sites within  $\alpha_{\text{M}}\beta_2$ , including the fact that one ligand (e.g., NIF) is capable of blocking the interaction of multiple ligands (C3bi, ICAM-1, fibrinogen) with the receptor (15, 45). However, although the binding sites for these ligands might be overlapping, they need not be identical (16, 17). Ustinov and Plow (22) have proposed a mosaic model in which many of the same loops and helices of the  $\alpha_{\text{M}}$ I domain, on or near its MIDAS face, may engage ligands, but different amino acid residues within these structures may contact the ligands. Direct support for this mosaic model is seen within the K<sup>245</sup>–R<sup>261</sup> segment of the  $\alpha_{\text{M}}$ I domain, which has been implicated in both NIF and fibrinogen recognition (23). Key contact amino acids in this loop for fibrinogen binding are F<sup>246</sup>, D<sup>254</sup>, and P<sup>257</sup>, whereas Y<sup>252</sup> and E<sup>258</sup> are involved in NIF binding. This same  $\alpha_{\text{M}}$ I domain segment is also involved in C3bi recognition by  $\alpha_{\text{M}}\beta_2$  (17).

Under the experimental conditions used in this study, which assayed the adhesion of activated neutrophils to surface-adherent platelets after vigorous washing, the predominant interaction between neutrophils and platelets appeared to be mediated by  $\alpha_{\text{M}}\beta_2$  binding to sGP Ib $\alpha$ , based on the ability of M2 to inhibit >80% of neutrophil adhesion. The enhanced potency of M2 with respect to inhibiting neutrophil adhesion to platelets (IC<sub>50</sub> = 30 nM) compared with BIAcore (kD = 17  $\mu$ M) is possibly secondary to the fact that leukocytes express  $\alpha_{\text{M}}\beta_2$  and platelets express GP Ib $\alpha$  in their native conformations. In contrast, BIAcore experiments used sGPIb $\alpha$  with immobilized M2 peptide.

Our data do not rule out the possibility of additional platelet surface receptors for  $\alpha_{\text{M}}\beta_2$ . Other potential  $\alpha_{\text{M}}\beta_2$  ligands present on the platelet membrane include fibrinogen (bound to GP IIb-IIIa; 34, 35), ICAM-2 (46), high molecular weight kininogen (38), and JAM-3 (43). However, a leukocyte-platelet interaction mediated by fibrinogen bridging between  $\alpha_{\text{M}}\beta_2$  and GP IIb/IIIa has been discounted by Ostrovsky et al. (47), who found that neither RGDS peptides nor the replacement of normal platelets with thrombasthenic platelets (i.e., lacking GP IIb/IIIa) affected the accumulation of the leukocytes on platelets. Although  $\alpha_{\text{M}}\beta_2$  binds ICAM-1, this receptor is not found on platelets. Platelets express a related receptor, ICAM-2 (48), but Diacovo et al. (5) have shown that ICAM-2 blockade has no effect on the firm adhesion of neutrophils on monolayers of activated platelets under flow. Santoso et al. (43) have reported recently that  $\alpha_{\text{M}}\beta_2$  may also bind to platelet

JAM-3, cooperating with GP Ib $\alpha$  to mediate neutrophil-platelet adhesive contacts (43).

The present observations also suggest a possible target for therapeutic intervention. In particular, the specificity of M2 inhibitory action toward GP Ib $\alpha$  (i.e., noninhibitory toward fibrinogen) suggests that it might be possible to prevent leukocyte attachment to platelets by targeting GP Ib $\alpha$  without inhibiting other  $\alpha_{\text{M}}\beta_2$  functions. Our recent observations have identified  $\alpha_{\text{M}}\beta_2$  as a molecular determinant of neointimal thickening after experimental arterial injury that produces endothelial denudation and platelet deposition. We found that antibody-mediated blockade (49) or selective absence (50) of  $\alpha_{\text{M}}\beta_2$  impaired transplatelet leukocyte migration into the vessel wall, diminishing medial leukocyte accumulation and neointimal thickening after experimental angioplasty or endovascular stent implantation. Therefore, this study identifying the precise binding site responsible for  $\alpha_{\text{M}}\beta_2$ -GP Ib $\alpha$  interaction might provide a molecular strategy for disrupting leukocyte-platelet complexes that promote vascular inflammation in thrombosis, atherosclerosis, and angioplasty-related restenosis.

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*Note added in proof.* Hoffmeister et al. (Hoffmeister, K.M., E.C. Josefsson, N.A. Isaac, H. Clausen, J.H. Hartwig, and T.P. Stossel. 2003. Glycosylation restores survival of chilled blood platelets. *Science*. 301: 1531–1534.) have recently reported that chilled platelets are cleared by hepatic macrophage  $\alpha_{\text{M}}\beta_2$  receptors via an interaction between platelet GP Ib $\alpha$  and a non-I-domain, lectin-binding site within  $\alpha_{\text{M}}\beta_2$ .

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